(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00233 A1

(51) International Patent Classification[7]: A61K 39/00, 38/16, 47/36, 9/14

(21) International Application Number: PCT/KR99/00466

(22) International Filing Date: 19 August 1999 (19.08.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1999-24336 25 June 1999 (25.06.1999) KR

(71) Applicant *(for all designated States except US)*: KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY [KR/KR]; 39-1, Hawolkok-dong, Sungbuk-ku, Seoul 136-130 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JEONG, Seo, Young [KR/KR]; 205-501, Mooncheon Village, Juyeob-dong, Ilsan-ku, Koyang-si, kyoungki-do 411-370 (KR). KWON, Ick, Chan [KR/KR]; #706-704, Cheongsol Apt., Hakae-dong, Nowon-ku, Seoul 139-230 (KR). PARK, Joo, Ae [KR/KR]; #102-1807 Sangyong Apt., Poongnab-dong, Songpa-ku, Seoul 138-040 (KR).

(74) Agent: LEE, Won-Hee; Suite 805, Sung-ji Heights II, 642-16, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

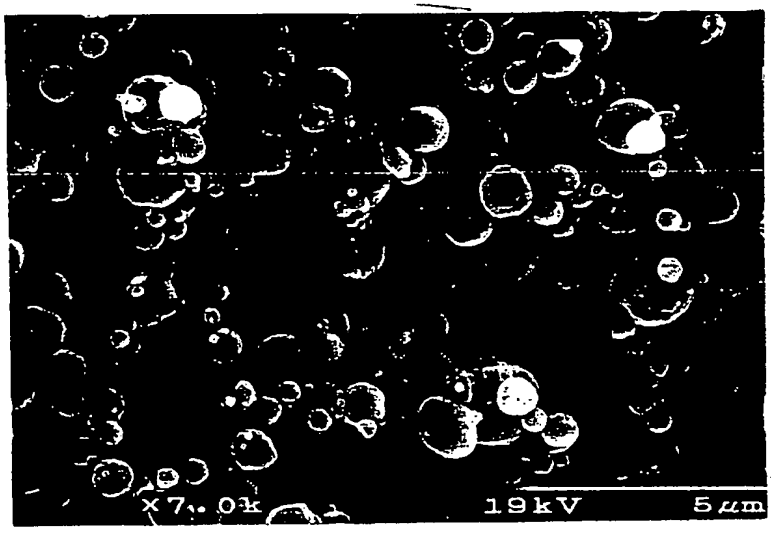
(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

[Continued on next page]

(54) Title: VACCINE COMPOSITIONS USING ANTIGENS ENCAPSULATED WITHIN ALGINATE MICROSPHERES FOR ORAL ADMINISTRATION AND PREPARATION PROCESS THEREOF



(57) Abstract: The present invention relates to vaccine compositions for oral administration consisting essentially of a protein antigen, in an amount effective to induce an immune response to said antigen, encapsulated in alginate microspheres, and to preparation process thereof. More particularly, the present invention relates to vaccine compositions for oral administration consisting of a protein antigen encapsulated in biodegradable alginate microspheres by the diffusion-controlled interfacial gelation technique which produces microspheres having less than 5 μm of diameter, and preparation process thereof.

WO 01/00233 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Vaccine compositions using antigens encapsulated within alginate microspheres for oral administration and preparation process thereof

FIELD OF THE INVENTION

The present invention relates to vaccine compositions for oral administration consisting of an protein antigen encapsulated in biodegradable alginate microspheres by the diffusion-controlled interfacial gelation technique which produces microspheres having less than 5 μm of diameter, and to preparation process thereof.

BACKGROUND

In the infection of human body, one of the major entry sites of pathogen is mucosal surface(Cundell, 1995). Therefore, immunization at the mucosal surface is an important alternative route for potential vaccines against pathogen infections. Also, this strategy could be beneficial to infants and elderly who have a deficient systemic immunity. Mucosal immune system is capable of responding to invading pathogens in the mucosal surfaces by producing pathogen specific secretory IgA antibodies. The local secretory IgA has been known to prevent both the colonization of pathogens at the mucosal tissues and the spread into the systemic circulation more efficiently compared to

the systemic antibodies(Service, 1994).

The mucosal tissues and secretory glands represent the largest residence of T cells, B cells, and plasma cells in the body. Additionally, secretory IgA, comprising more than 60% of all immunoglobulins in the body, is produced in these tissues. Stimulation of mucosal immunity is believed to arise at specialized aggregates of lymphoid tissues collectively termed the bronchus-associated lymphoid tissue(hereinafter, referred to as "BALT") and gut-associated lymphoid tissue(hereinafter, referred to as "GALT"). Unique characteristics of BALT and GALT include the ability to communicate immunogenic information arising at one mucosal surface to other mucosal surfaces in the body. Furthermore, it has been reported that, if properly modulated, GALT and BALT could confer systemic immunity as well as mucosal immunity against diverse toxins and pathogens(Shalaby, 1995).

Despite the importance of mucosal immunity in protection, immunization using mucosal pathway has been generally ignored in the past. Vaccines administered parenterally induce poor mucosal immunity. It may be due to the functional separation of the systemic and mucosal immune systems. As a new approach to overcome this problem, mucosal vaccines using proper delivery systems seem to provide promising solutions.

Mucosal immunity is characterized in the

predominance of dimeric IgA in external secretions and the preponderance of IgA secreting plasma cells beneath secretory epithelia. Mechanisms of antibacterial function of secretory IgA are based on its opsonizing properties and its ability to interfere with the adherence of bacterial antigens to mucosal surfaces(Abraham, 1994), thereby limiting bacterial colonization and enhancing elimination of bacteria. Other mechanism is reported that the secretory IgA may lyse bacteria in the presence of lysozymes and complements. The production of IgA is initiated at specialized lymphoid tissues, in particular those in the upper respiratory and gastrointestinal tracts. The latter tissues are called GALT and consist of Peyer's patches(hereinafter, referred to as "PP"), appendix and solitary lymphoid nodules. BALT, whose most prominent follicles are the palatine and pharyngeal tonsils, is the principal mucosal lymphoid tissue of the respiratory tract. It should be noted however, that there are some differences in functions of BALT and GALT.

In the dome region of PP, there is a specialized epithelium known as microfold or M cells, which pick up antigens and transport them into underlying lymphoid tissues. Antigens are processed and stimulate B and T cells in the germinal centers of follicles located beneath the dome. These cells leave the PP via the

efferent lymphatics and enter the systemic circulation through the thoracic duct(see Fig.1). Especially, the B cells expand clonally in the mesenteric lymph node(MLN) and become mature IgA plasma cells, which subsequently produce secretory IgA in external secretions. This cellular distribution system comprises the common mucosal immune system of the body. One of the evidences on the existence of a common mucosal system is that specific secretory IgA induced by oral immunization is detected in peripheral blood and mucosal tissues by remote secretions(Czerkinsdy *et al.*, 1991).

To generate effective and protective immune responses by the mucosal route, novel strategies have been employed.

One strategy is the incorporation of a powerful adjuvant such as cholera toxin(CT), *E.coli* heat-labile endotoxin, muramyldipeptide or phorbol ester into vaccine formulation. These mucosal adjuvants seem to work by modulating the signal transduction pathway of immune cells or by stimulating the immune responses of microenvironment. Among them, CT is the most potent mucosal adjuvant identified up to now. Its immunomodulating effects include an enhanced antigen presentation by a variety of cell types, promotion of isotype differentiation in B cells leading to an

increased IgA formation and complex stimulatory as well as inhibitory effects on T-cell proliferation and lymphokine production(Holmgren *et al.*, 1993). Although the precise mechanism in the actions of cholera toxin has not been proved yet, the adjuvant actions appear to be closely related to the binding property of cholera toxin B subunit to the GM-1 ganglioside and the ADP-ribosylation activity of A subunit of the toxin.

An altenative strategy is to modify the physical form of the antigen. For example, antigens can be incorporated into liposome with micrometer or nanometer size, microemulsion, microspheres or nanospheres by microencapsulation method using colloidal microparticle.

Microencapsulation is defined as a technique whereby chemical or biological materials such as drugs, enzymes, toxins, bacteria and viruses are encapsulated within polymer matrices, which has advantages preventing the encapsulated materials from being inactivated by water, heat or oxidation, or enhancing the biocompatibility of the materials. Especially, by using microspheres technology, antigens can be incorporated into microcapsules that protect antigens from denaturation at low pH and hydrolytic degradation by enzymes in the gastrointestinal tract. Additionally, antigen sequestration in microcapsules may provide a mechanism to evade neutralization by maternal antibody. Biodegradable and biocompatible

microspheres have been used for antigen delivery into the GI tract(Muir *et al.*, 1994).

Most of the methods to produce microspheres, however, include the steps of using organic solvent and/or heating at high temperature. Antigens may denature under these harsh conditions. The production of microspheres using gelatin, poly(D,L-lactic acid), poly(glycolic acid) or albumin, for example, may involve heat as high as 130 °C or the use of organic solvents such as formaldehyde, glutaraldehyde, methylene chloride and hexafluoroacetone sesquihydrate, to dissolve or to cross-link the polymer(Jepon *et al.*, 1993, Maloy *et al.*, 1994).

Unlike other microspheres, the alginate microspheres can be prepared easily in aqueous solutions at room temperature and, therefore, are very useful in encapsulating antigens.

Alginate is an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, and has been used as a food additive, compressed tablets disintegrator and gelation agent. Alginate also has been used widely applied to immobilize cells, to hybrid artificial organs and as delivery system for drugs and antigens due to its non-toxicity and biocompatibility (K.Park, 1993).

Alginate has an unique property of forming a gel in the presence of divalent cations such as Ca^{2+}. Such

a gel formation occurs mainly at the junctions between ions and homopolymeric blocks of guluronic acid. Since calcium-alginate gel produced through this process has bridge formed by ion bond, it can make hard hydrogel. Alginate gel can be prepared easily in aqueous solutions and also swelled and gradually disintegrated in a living body. Therefore, alginate has been widely used for the polymer matrix of microspheres owing to the physiochemial properties required for polymer matrix.

However, the current technology could not make alginate microspheres smaller than 10 μm in diameter thus far.

It is well known that the uptake of microspheres at the small intestine is restricted to microspheres of less than 10 μm in diameter(Jepson, 1993). Kinetic studies on the fate of the microspheres within the GALT showed that the microspheres small than 5 μm in diameter were transported through the efferent lymphatics, while those larger than 5 μm in diameter remained in PP.

In conventional methods, small sized alginate microspheres had been prepared by using fluidized bed and rotating pans. However, the gels produced by adding alginate droplets into the aqueous solutions containing calcium ions have a large mean size and

broad size distribution. Therefore, new procedures whereby microspheres can be produced with a desirable size range are required to deliver antigens to the PP.

Streptococcus pneumoniae is a major respiratory mucosal pathogen that causes meningitis, otitis media, bacteremia, and pneumonia. It has been well established that specific antibodies to capsular polysaccharides can elicit a protective immune response against pneumococcal infection in adults(Macleod, 1945). In the case of children(<2 years), elderly, and those with immunodeficiencies, however, the polysaccharide vaccines do not elicit effectively pneumococcus-specific protective antibody responses(Anderson, 1977, Peters, 1994) and children can have repeated infections involving strains of the same capsular serotype. Protein polysaccharide conjugates or pneumococcal proteins alone have been considered as an alternative means to induce protective immunity in infants and children against pneumococcal infection(Yamamoto, 1997).

The immunogenic nature of proteins makes them prime targets for new vaccine strategies. Pneumococcal proteins being investigated as potential vaccine candidates include pneumolysin, neuraminidase, autolysin, pneumococcal surface adhesion A(PsaA), and pneumococcal surface protein A(PspA)(McDaniel, 1996). These proteins could become more efficacious vaccines

since they would offer a broader range of protection against a greater number of serotypes and could elicit T cell dependent immune response and also provide a memory response.

Among the reported pneumococcal proteins, only pneumolysin and the pneumococcal surface protein A(PspA) have been extensively examined as vaccine candidates. Both proteins, known to give a partial protection in mice, are promising putative vaccine candidates(AlonsoDevelasco, 1995).

We, the inventors of the present invention, developed a novel vaccine compositions for oral administration consisting of a protein antigen encapsulated in biodegradable alginate microspheres by the diffusion-controlled interfacial gelation technique and preparation process thereof.

Alginate-encapsulated antigen of this invention shows higher levels of antigenicity than the naked protein antigen, and are considered as a proper carrier for an efficient delivery of antigens by the Peyer's patch and by concomitant transport through lymphatics because of having less than 5 μm of diameter.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a vaccine composition for oral administration consisting essentially of a protein antigen, in an amount effective to induce an immune response to said antigen, encapsulated in alginate microspheres.

It is a further object of this invention to provide the vaccine composition wherein the size of the alginate microspheres is between 0.1 to 5 μm.

It is an additional object of this invention to provide the vaccine composition causing mucosal immune response.

It is also an object of this invention to provide a process for preparing the vaccine compositions comprising the steps of:

a) Mixing aliquot of a protein antigen or a protein antigen including immune adjuvant with a alginate aqueous solution;

b) Homogenizing by adding a mixture of the alginate and a protein antigen of step a) to n-octanol containing an emulsifier;

c) Spraying n-octanol solution containing $CaCl_2$ into the emulsion while stirring the whole emulsion slowly;

d) Adding additional $CaCl_2$ solution to saturate the emulsion, and curing microspheres;

e) Dehydrating microspheres by addition of dehydrating solvent;

f) Collecting the microspheres on membrane filters and washing with alcohol, and then drying *in vacuo*.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides vaccine composition wherein the protein antigen is selected from the group consisting of bacterial surface proteins, endotoxin or exotoxin antigens, autoantigens, and allergens. Besides, various protein antigens can be used in vaccine composition of this invention; i.e., pnemococcal antigen, *Hemophilus parainfluenza* antigen, diphtheria antigen, pertussis antigen, tetanus antigen, enterotoxigenic *E. coli* antigen, dysentery antigen, cholera antigen, gonococcus antigen, influenza virus antigen, B type hepatitis antigen, measles antigen, smallpox virus antigen, and rubella antigen. Preferably, it can be selected from pneumococcal proteins such as pneumolysin, neuraminidase, autolysin, pneumococcal surface adhesion A(PsaA) and pneumococcal surface protein A(PspA).

This invention also provides the vaccine composition including additionally immnune adjuvants, such as cholera toxin, cholera toxin B subunit, *E. coli*

heat-labile enterotoxin, muramyldipeptide or phorbolester, added to inside or outside of microspheres.

In addition, this invention provides a process for preparing the vaccine composition, wherein the diffusion-controlled interfacial technique is employed.

Further features of the present invention will be disclosed hereinafter in detail.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows circulation of lymphocytes within the mucosa-associated lymphoid system.

Fig. 2a shows the structures of alginate monomers, β-D-mannuronic acid(M) and α-L-guluronic acid(G).

Fig. 2b shows a proposed structural model for the chelation of ions by GG residues and the gel formation by binding of divalent cation such as Ca^{2+}.

Fig. 3 shows fabrication method to prepare alginate microspheres.

Fig. 4 shows construction process of plasmid pCYB4-PsaA.

Fig. 5 shows the result confirming expression of recombinant protein by 7 clones using western blotting.

Fig. 6 shows nucleotide sequence of PsaA gene.

Fig. 7 shows growth curve of transformed XL_1-Blue

at different temperatures.

Fig. 8 shows differences in band patterns in pellets and supernatants of cell lysates at various culture temperatures by western blotting.

Fig. 9a shows the identification of the purified PsaA(35 kDa) protein on SDS-PAGE.

Fig. 9b shows the identification of the purified PsaA(35 kDa) protein by western blotting.

Fig. 10 shows *in vitro* release profile of the entrapped PsaA from alginate microspheres.

Fig. 11 shows immunogenicity of recombinant PsaA introduced by subcutaneous immunization.

Fig. 12 shows standard curve for the quantification of antibodies determined by a sandwich ELISA.

Fig. 13 shows antibody responses induced by oral immunization with encapsulated PsaA[hereinafter, referred to as E(PsaA)] and naked PsaA[hereinafter, referred to as N(PsaA)].

Fig. 14 is an electromicroscopic photograph which shows that alginate microspheres of the present invention are smaller than 5μm in diameter.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the notion that vaccine composition for oral administration may be

prepared through process that protein antigen is encapsulated in biodegradable alginate microspheres by the diffusion-controlled interfacial gelation technique which produces microspheres having less than 5 μm of diameter.

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides vaccine composition wherein protein antigen is selected from the group consisting of bacterial surface protein antigens, endotoxin or exotoxin antigen, autoantigens, and allergens. Besides, various protein antigens can be used in vaccine composition of this invention; i.e., pnemococcal antigen, *Hemophilus parainfluenza* antigen, diphtheria antigen, pertussis antigen, tetanus antigen, enterotoxigenic *E. coli* antigen, dysentery antigen, cholera antigen, gonococcus antigen, influenza virus antigen, B type hepatitis antigen, measles antigen, smallpox virus antigen, and rubella antigen. Preferably, it can be selected from pneumococcal proteins such as pneumolysin, neuraminidase, autolysin, pneumococcal surface adhesion A(PsaA), and pneumococcal surface protein A(PspA).

Especially, pneumococcal surface adhesion A(PsaA) proteins of *Streptococcus pneumoniae* is preferred as antigen encapsulated in alginate microspheres, and can

be obtained by process comprising the steps of;

a) Amplifying the gene encoding PsaA by the polymerase chain reaction(PCR) using the chromosome DNA of *S.pneumoniae* as template;

b) Cloning the PsaA gene into pCYB4 vector;

c) Transforming *Escherichia coli* with the recombinant vector;

d) Incubating the *Escherichia coli* transformant; and

e) Isolating and purifying the recombinant PsaA

Alginate microspheres including protein antigens can be prepared easily in aqueous solutions at room temperature, and have advantages such as non-toxicity and biocompatibility. Therefore, alginate, an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, is of great use for encapsulating antigens.

In addition, the vaccine composition of this invention includes additionally immnune adjuvants, such as cholera toxin, cholera toxin B subunit, *E. coli* Heat labile enterotoxin, muramyldipeptide or phorbolester, which are added to inside or outside of microspheres to increase the immunogenicity.

The preferred size of the alginate microspheres is less than 5 μm of diameter to effectively deliver antigens. The smaller the size of alginate microsphere is, the lower the toxicity and carcinogenicity are,

because of the decreased stimulation in the target sites. Therefore, the present invention provides the method for preparing small and homogeneous particles by water-in oil emulsion technique, not by gelation technique in the interface between two aqueous layers.

In another aspect of this invention, also provided is a process for preparing the vaccine compositions comprising the steps of:

a) Mixing aliquot of a protein antigen or a protein antigen including immune adjuvant with a alginate aqueous solution;

b) Homogenizing by adding a mixture of the alginate and a protein antigen of step a) to n-octanol containing an emulsifier;

c) Spraying n-octanol solution containing $CaCl_2$ into the emulsion while stirring the whole emulsion slowly;

d) Adding additional $CaCl_2$ solution to saturate the emulsion, and curing microspheres;

e) Dehydrating microspheres by addition of dehydrating solvent;

f) Collecting the microspheres on membrane filters and washing with alcohol, and then drying *in vacuo*.

The alginate microspheres solution of step a) is used preferably in the concentration of between 1-5 weight%. The ratio of the alginate aqueous solution to

total solvent volume between 1:1 to 1:10 is preferred, which controls the size and the gelation of microspheres.

As for the emulsifier of step b) hydrogenated castor oil(hereinafter, referred to as "HCO")-10, HCO-60 and Span-80 are preferred of which the concentration is between 1-10 weight%.

Also, the n-octanol containing CaCl of step c) is used preferably in the concentration of between 0.5-2 weight%, and the $CaCl_2$ solution of step d) between 5-10 weight%.

As for the dehydrating solvent of step e), methanol, ethanol, isopropanol or acetone is preferred. The filter of step f) is preferably a polyvinylidene difluoride(PVDF) membrane filter with a pore size of between 0.10-0.44 μm.

On the size of microspheres, the effect of surfactant is also important. As the polarity of the surfactant increases, by increasing the polyethylene oxide(PEO) units of hydrogenated castor oil(HCO) series, the size of microspheres decreases.

Besides, various preparation parameters can affect the particle size distribution.

The initial concentration of alginate in the aqueous phase is one of these parameters. At the concentration higher than 5%, it is difficult to

produce small and homogeneous microspheres since the viscosity of the alginate solution is too high. On the other hand, at the concentration lower than 5%, agglomerated microspheres are obtained.

The choice of the organic solvent is found to be another important factor in determining the particle size and its distribution. Throughout the distribution polymerization studies, it was shown that the particle size decreased as the solvent polarity increased (Lok and Ober, 1985, Paine, 1990). In this invention, n-octanol is used as an organic solvent in which $CaCl_2$ is dissolved and the solution is slowly diffused into the aqueous phase containing alginate for the diffusion-controlled interfacial gelation.

The stirring rate is also an important factor in forming emulsion droplet with a desired diameter. In a preferred embodiment of this invention, the emulsification was performed at 8,000 rpm for one hour. When the stirring speed was lower than 5,000 rpm, heterogeneous microspheres with diameter larger than 10 μm are obtained. On the other hand, a high speed stirring above 10,000 rpm resulted in the formulation of agglomerated particles with a low yield. A possible the stirring rate is about 5,000 rpm to about 10,000 rpm, and is 7,000 to 8,000 rpm preferably. In addition, further continuous stirring at a low speed is required until the gelation is completed so that the hardened

microspheres could be isolated as discrete particles.

The particle size is also dependent on the concentration of $CaCl_2$ in n-octanol and the rate of addition into the medium. The size of the microspheres could be further reduced by spraying $CaCl_2$ solution onto the emulsion rather than by dropwise addition using a syringe.

The microspheres used for an *in vitro* release test in a preferred embodiment of this invention, are prepared by 5%(w/v) alginate solution emulsified in a n-octanol solution containing 5% HCO-60, followed by curing with 1% $CaCl_2$ in n-octanol.

Surface morphology and size of microspheres loaded with antigen are observed by the scanning electron microscopy. The microspheres of this invention has a smooth surface without noticeable defects, with the diameter in the range of 0.5 to 5 μm.

The Alginate microspheres of this invention may be administered orally to human, used as the preparations of various drug forms. The preparations for oral administration includes compressed tablets, pills, pulves, granules, capsules *et al.*, and may contain appropriate one or more excipients such as starch, calcium carbonate, sucrose, lactose or gelatin. Besides additives, lubricants such as a magnesium stearate, talc may be added.

In using the Alginate microspheres of this invention as vaccine compositions for oral administration, the effective dose of protein antigen is 20-400 μg/dose, and preferably 40-100 μg/dose.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

<Example 1> Cloning of the PsaA gene from chromosome of *S.pneumoniae*

(1-1) Isolation of genomic DNA from *S.pneumoniae*

S.pneumoniae was cultured in BHI and 10% FBS media by anaerobic incubation at 37°C overnight. Cultured *S.pneumoniae*(1.5ml) was harvested by centrifugation for 2 min at 13,000rpm in a microcentrifuge. The pellet was resuspended in 340μl TE buffer containing 27 μl of lysozyme(10 mg/ml) and incubated at room temperature for 30 min. After addition of 30μl of 10 % SDS and 3 μl of proteinase K(20 mg/ml), the mixture was incubated

at 37 °C for 1 hr, and 100 μl of 5 M NaCl and 80 μl of CTAB/NaCl(10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) solutions were added and the mixture was incubated at 65 °C for 10 min. DNA was extracted with an equal volume of chloroform/isoamyl alcohol, and precipitated by a standard protocols(Maniatis, 1989).

(1-2)Preparation of pCYB4 vector

pCYB4(6.8kb, New England Biolabs, USA) contains the IPTG-inducible Ptac promotor and a multiple cloning site(MCS). The vector was designed to produce a fusion of the C-terminus of the target protein and the N-terminus of the intein. The DNA encoding chitin binding domain(CBD) is present at the C-terminus of the intein for affinity purification. *NcoI* and *SmaI* restriction enzyme(Promega, USA) sites are present at MCS. Recombinant plasmid was transformed into *E.coli* XL_1-Blue(Novagen, USA) by electroporation, and plasmid DNA was prepared according to the standard protocol to give the genomic DNA of *S.pneumoniae*(Maniatis, 1989).

(1-3) Cloning of the PsaA gene

DNA sequence for PsaA was retrieved from DNA database as shown in Fig. 6. The gene PsaA encodes a 37-kDa protein of *S.pneumoniae*. The reported 2.4kb fragment has three open reading frames(ORF). ORF 1 and 3 flank PsaA gene. ORF 2, which is identified as psaA

of 933bp, encoding a Mr 34, 541 protein of pI 5.93. Although mature PsaA protein is hydrophilic, it has a hydrophobic leader sequence of 20a.a.. PsaA gene was amplified by polymerase chain reaction(PCR), and analyzed by agarose gel electrophoresis. The 877bp PCR product was purified from the gel by Qiagen elution Kit.

The amplified region by PCR in the total 1330bp gene is between position 232 and 1112. Signal peptide and stop codon were removed from each terminus. Reaction mixture(50 μl) contained 2 units of Taq polymerase, 400 nmol of a forward primer(SEQ ID No;1), 400 nmol of reverse primer(SEQ ID No;2), 1 μg of genomic DNA as a template and 200 μM of each dNTP. Primers for PCR were synthesized by Oligos Etc.Inc.(Willsonile, USA).

Thermal cycle for amplification was : 1 cycle of 94 °C for 3 min; 32 cycles of 94 °C for 15 sec, 55 °C for 15 sec, 72 °C for 1 min; final extension at 72 °C for 5 min and holding at 4 °C. After PCR, the reaction mixture was electrophoresed and then eluted from an agarose gel by QIA quick Gel Extraction Kit. The resulting DNA pellet was dissolved in distilled water. The DNA was incubated at 45 °C for 15 min in a Klenow enzyme(2 unit/1 μl) buffer in twice the volume of the DNA solution and followed by adding 1/10 volume of total reaction volume for 0.125 mM dNTP at 37 °C

for 30 min. The mixture was treated with TE-saturated phenol followed by chloroform: isoamylalcohol(24:1 by vol), and DNA was precipitated with ethanol. Finally, the eluted DNA was dissolved in distilled water.

Amplified insert DNA and pCYB4 vector were digested first by *SmaI* at 25 °C and secondly by *NcoI* at 37 °C. Digested DNA was electrophoresed, eluted by QIAquick Gel Extraction Kit, and was treated with TE-saturated phenol and chloroform:isoamylalcohol(24:1), followed by the ethanol precipitation. Purified DNA was redissolved in distilled water.

The vector and insert DNA were mixed thoroughly by pippetting and incubated at 45 °C for 15 min to solubilize DNA, followed by storage on ice for 5-10 min. T4 ligase and 1 mM ATP were added into the mixture in a ligase buffer. Ligation reaction was performed at 4 °C overnight(see Fig.4).

(1-4) Transformation

Preparation of competent *E.coli* was performed as follows: *E.coli* XL_1-Blue were inoculated in 10 ml SOB(2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) media supplemented with tetracycline(50 ug/ml) and were cultured overnight. One microliter of culture cells ($1.6X10^9$ cell/ml) was transferred to 100 ml of fresh

SOB/TC and grown at 37 °C in a shaking incubator. When OD_{600} reached 0.5, the culture was put on ice for 20-30 min. Then the cells were harvested by centrifugation (Sovall SS-34 rotor: 3,000 rpm, 15 min, 4 °C). After discarding the supernatant, the pellet was resuspended in 10 ml of ice-cold RF1[100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$ and 15%(w/v) glycerol(pH5.8)] solution, and placed on ice for additional 15 min. Cells were harvested as above, and the pellet was resuspended in 8 ml of ice-cold RF2[10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$ and 15 %(w/v) glycerol(pH6.8)] solution. The cell suspension was aliquoted(100 μl per tube) and each aliquot was mixed with 4 μl of ligation mixture obtained from the step (1-3). The DNA-cell mixture was chilled on ice for 1 hr and incubated at 42 °C for 2.5 min, followed by 2 min incubation on ice again. Fresh LB(500 μl) was added to the mixture followed by incubation at 37 °C for 1 hr without shaking. Aliquot(100-200 μl) of this mixture was plated on a LB agar plate containing ampicillin(100 ug/ml). The plate was incubated at 37 °C.

(1-5) Screening for transformants

To identify colonies harboring pCYB4-psaA plasmid, plasmids were purified from each colony and digested

with restriction enzyme. The control colony was a transformed XL_1-Blue cell harboring only pCYB plasmid. To begin with, 32 colonies were selected out of total 178 colonies. Some colonies from the selected 32 colonies were randomly picked up, and was inoculated in LB media containing ampicillin. Each plasmid was purified from the culture as described in step (1-2). Purified plasmids were double-digested by *KpnI* and *NcoI* at 37 °C into pCYB4 vector and PsaA insert.

To see whether the clones could actually express PsaA-intein/CBD fusion protein, preliminary expression test was performed. Each clone showing to have the pCYB4-PsaA plasmid by double digestion of purified plasmid was inoculated into 5 ml LB supplemented with 100 μg/ml of ampicillin and cultured overnight. An aliquot(50 μl) of the overnight culture was transferred to 5 ml of fresh LB containing ampicillin and grown at 37 °C for 3 hrs in a shaking incubator. Cultures were induced with or without 500 μM IPTG(isopropyl-β-D-thiogalactoside, Boehringer Manheim, Germany) for additional 2 hrs at 37 °C. Each culture was centrifuged and resuspended, cell pellet was sonicated, and cell lysate was analyzed on SDS-PAGE and by western blotting.

SDS-PAGE and western blotting were performed as described by Maniatis *et al*(1989). Samples were heated

in a boiling water bath for 3 min and separated in 10 % polyacrylamide gel. The gel was stained with Coomassie brilliant Blue R250 and dried by using a gel drying kit (Promega, Madison, WI, USA). For the western blotting, proteins were transferred from the gel to polyvinylidenefluoride(PVDF) membrane(Amersham, UK). Anti-PsaA rabbit serum was diluted 100 folds in PBS and used as a primary antibody. Peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies(Organon Teknika Corp, USA) were also diluted in PBS(1:10,000) and used as secondary antibodies. For the color development, washed membrane was incubated in a 4-chloro-1-naphtol (Sigma chemical Co., USA). As a result, 4 clones expressed either 90 or 35 kDa proteins.

(1-6) DNA Sequencing

Cloned PsaA gene sequence was confirmed by DNA sequencing. The first half of total 877 bases was determined by extension of Ptac promotor primer and the latter half by extension of the intein reverse primer. Sequencing was performed by dideoxy chain termination method using automatic fluorescence sequencer(Applied Biosystems, Model 373)(Sanger *et al.*, 1997). All procedures were done following the Manufacturer's instruction. 'Forward' reaction mixture(20 μl) contained 5 pmol 1 of Ptac promotor primer(SEQ ID No;3), 1 μg of template plasmid and 8 μl of Thermo

Sequenase™ dye terminator cycle sequencing pre-mix kit (Amersham Life Science, UK). 'Reverse' reaction mixture(20 μl) contained the same components as the 'Forward' mixture except that 5 pmols of the intein reverse primer(SEQ ID No;4) was used in place of the Ptac promotor primer. The condition for PCR was: 30 cycles of 96 °C for 30 sec, 45 °C for 15 sec, 60 °C for 4 min. After PCR, the mixture was transferred to a fresh tube containing 2 μl of 3 M ammonium acetate and 50 μl of 95 % ethanol, then chilled at -70 °C for 10 min followed by centrifugation. The pellet was washed with 70 % ethanol and was dried in a SpeedVac. Then DNA was redissolved in 4 μl of loading buffer (deionized formamide and 50 mM EDTA(pH8.0) in a ratio of 5:1, heated at 70 °C for 5 min and placed immediately on ice. DNA was resolved on a 6% acrylamide gel containing 6 M Urea in 1x TBE buffer(90 mM Tris-borate, 2 mM EDTA). Recombinant clones which were confirmed to be positive by DNA sequencing and expression of recombinant protein were grown in 5 ml of LB containing 100 μg/ml ampicillin. When OD_{600} of the culture reached 0.5-0.6, 800 μl of culture was mixed with 200 μl of glycerol. The mixture was stored at -70 °C as a cell stock.

<Example 2> Expression and purification of recombinant PsaA proteins

(2-1) Expression of the recombinant PsaA protein

A fresh colony of clone selected from step (1-5) was inoculated into 10 ml LB media containing ampicillin and incubated at 37 °C overnight. Cultured cells were seeded into 1 liter of fresh LB medium containing ampicillin and incubated at 37 °C in a shaker at 600 rpm for 3 hrs. When OD_{580} reached 0.5-0.6, culture was induced with 0.5 mM IPTG and incubated at 20 °C for additional 18 hrs until OD_{580} reached 2.0-2.1. The cells were harvested by centrifugation at 8,000 rpm for 30 min at 4 °C, using Sovall SLA-1500 rotor. Cell pellet was stored at -70 °C until the next experiments.

Each pellet and supernatant were analyzed on SDS-PAGE and by western blotting(see Fig.8).

(2-2) Purification of the recombinant PsaA protein

The pellet was resuspended in 25 ml ice-cold column buffer(Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA and 0.1 % Triton X-100). The cells were lysed by addition of 1.5 ml of lysozyme(10 mg/ml) and sonication. The resulting lysate was centrifuged at 14,000 rpm for 30 min at 4 °C, using Sovall SS-34 rotor. Supernatant was loaded onto the chitin beads affinity column pre-equilibrated with the same buffer. The chitin beads have a binding capacity of about 2 mg protein/ml. Purification of recombinant PsaA protein was performed

as Manufacturer's 'IMPACT™I: One step Protein Purification System Manual'. The sample was reloaded 3 times onto pre-equilibrated column at 4 °C. The column was washed with column buffer(30x volume of chitin bead slurry) and was flushed with 3 volume cleavage buffer(Tris-HCl, pH8.0, 500 mM NaCl, 0.1 mM EDTA and 30 mM DTT). When DTT(dithiotreitol) was distributed throughout the column, the flow was stopped and the column was left at 4 °C overnight after tight sealing. On the next day, PsaA protein was eluted with 3 volumes of column buffer. The result shows that PsaA protein was eluted and Intein/CBD protein was left in column. The size of purified PsaA protein analyzed by SDS-PAGE and western blotting was about 35 kDa, which was consistent with the native PsaA protein(see Fig. 9a and 9b).

The purified PsaA protein was concentrated to 1.5 mg/ml using Amicon Centriprep©(molecular weight cut off: 10,000). This concentrate was dialyzed to remove DTT against PBS or distilled water and used directly or as an encapsulated antigen, respectively. Antigens were stored at -20 °C until use. The Protein content was determined by Bradford assay kit(Bio-Rad, USA). The yield of pure PsaA was 10 mg per liter of bacterial culture.

<Example 3> Microencapsulation

(3-1) Preparation of alginate microspheres

The microspheres were prepared by a modified water in oil(w/o) emulsion technique(see Fig. 2a, 2b and 3). Alginate solution[5 ml, 5.0 %(w/v)]were added dropwise to 30 ml of n-octanol containing HCO-60[hydrogenated castor oil, 5 %(w/v)] as an emulsifier. The w/o emulsion was prepared by homogenizing the mixtures for 1 hr in 8,000 rpm with homogenizer(Ultra-turrax© disperser, IKA Werke, Germany) in a 100 ml baffled pyrex beaker(750mm in diameter x 900mm in height). Then, n-octanol containing 1 %(w/v) calcium chloride was added into the emulsions by an air sprayer(Fuso Seiki Co. Ltd., Japan) while stirring the whole medium slowly with a magnetic stirrer. The microspheres were cured for 10 minutes after mixing additionally with 5 ml of 8%(w/v) $CaCl_2$ to saturate the mixture. Then 6 ml of isopropyl alcohol was added slowly to dehydrate the microspheres. Finally, the microspheres were collected onto polytetrafluoroethylene(PTFE) membrane filters with pore of 0.44 μm(Alltech Associates, Inc. IL). The microspheres on the filter were washed with 20 ml of isopropyl alcohol and dried in vacuum for 18 hrs. The size and surface morphology of the microspheres were examined using a scanning electron microscope(S-2460N, Hitachi Ltd., Japan) as described elsewhere(Chun *et al.*, 1996).

As a result, the micropsheres had a smooth surface without noticeable defect, with the diameter in the range of 0.5 to 5 μm.

(3-2) Preparation of alginate microspheres containing antigens

Aliquots of antigens were added to 5 ml of 5% alginate aqueous solution to prepare the microspheres as described in step (3-1). The emulsion was prepared by homogenizing in 8,000 rpm for an hour. HCO-60(Nikko Chemical Co. Ltd, Japan) was used as a surfactant and 1% $CaCl_2$ solution was used for the gelation. After vacuum dry, the microspheres were stored in a sealed vial at 4 °C until use. The loading efficiency of antigens was measured by assaying the protein content. Total loaded amount of the antigens in the microspheres was determined by dissolving the microspheres completely in 0.1 M sodium citrate buffer at pH7.8 for 3 hours with magnetic stirring. After the dissolved solution was dialyzed against PBS, the total protein concentration was determined by the Bradford assay.

In result, The average efficiency for the encapsulation of PsaA protein [(encapsulated protein/total protein in the solution)x 100] was 24%(42% for BSA protein). The ratio of total PsaA protein loaded in the microspheres[(weight of protein loaded/weight of microspheres)x 100]was approximately

0.4%(w/w) and that of BSA protein loaded was 0.8 %(w/w). The low encapsulation efficiency for PsaA protein may be due to the low protein concentration in the solution and a small size of PsaA protein.

<Example 4> In vitro antigen release study

The rate of the PsaA release from microspheres was determined as follows. The known amount of microspheres(10 mg) was placed in sealed vials containing PBS(1 ml) and shaken at 37 °C. At various time points, aliqouts of supernatant were drawn after centrifugation at 1,000 g for 15 minutes and the protein content was determined by Bradford assay. The amount of protein in the aliquots was related to the total protein in the samples of microspheres and then, the cumulative percent release of protein was determined as a function of time.

A typical *in vitro* release profile of PsaA from the alginate microspheres in PBS is shown in Fig. 10. PsaA was continuously released for 18 hours after having a burst of the release for the first hour. Although about one-half of the encapsulated antigen could be lost during the initial phase, 50% of antigens left in alginate micropheres after the initial burst could be released in a sustained fashion.

<Example 5> Immunological assay

(5-1) Immunogenisity test of recombinant PsaA

Female Balb/c mice, 6 to 8 weeks old, were obtained from DAEHAN Laboratory Animal Research Center Co.(Korea). The animals were kept under standard specific pathogen free(SPF) conditions and given pelleted food and tap water throughout the experiment *ad libitum*. For systemic immunization, the emulsion of the antigens dissolved in PBS(200 μl) and complete Freund's adjuvant(boosting was performed with incomplete Freund's adjuvant) was injected subcutaneously(S.C.). The doses based on the PsaA contents were 40 μg/mouse for immunization. The mice were immunized in 2 week intervals for the second and third times.

Blood was collected from a puncture of the retroorbital plexuses in heparinized capillary pipettes 2 weeks after the third immunization and the mice were exsanguinated to collect gut wash and lung lavages. Serum was separated from whole blood following coagulation at 4 °C for 18 hrs by centrifugation. Gut samples were collected as described previously by Elson *et al* (1984). In brief, 1 ml of lavage fluid(25 mM NaCl, 40 mM Na_2So_4, 10 mM KCl, 20 mM $NaHCO_3$, 50 mM EDTA, 0.1 mg/ml soybean trypsin inhibitor, and 162 mg/ml polyethylene glycol(average molecular mass=3,350)) was injected to a isolated small intestine. After 10 min, intestine was squeezed and the fluid was collected into

microcentrifuge tube. After vigorous vortexing and centrifugation(1,000 X g, 10 min), supernatant was transferred to a microcentrifuge tube with 0.01 volume of 100 mM phenylmethylsulfonyl fluoride(PMSF, Sigma Co.). Samples were centrifuged for 15 min at 13,000 xg, and the supernatant was recovered and mixed with 0.01 volume of 100 mM PMSF. The clarified gut contents were placed on ice, and after 0.05 volume of 3.5 % globulin-free BSA solution was added, it was kept frozen at -70 °C until use. Bronchoalveolar lavage was collected by pertracheal cannulation and gently washed with 0.7 ml of ice-cold PBS. About 0.5 ml of lung lavage was recovered from each mouse, and stored at -20 °C after centrifugation 4 °C at 3,000 xg for 5 min to remove debris. The blood contamination of the bronchoalveolar lavage fluid was assessed by detecting hemoglibin at $O.D._{575nm}$. The murine blood diluted serially in PBS served as a standard. The mean blood contamination was 4± 0.7 %. PsaA specific antibody responses at serum, gut wash and lung lavage were assayed according to the method of step (5-2).

Anti-PsaA antibodies formed by immunization which was executed for the purpose of confirming the immunogenicity of recombinant PsaA is shown in Fig.11. At 2 weeks after third immunization, the levels of anti-PsaA antibodies of IgG and IgA increased generally, but the extent of increment was locally different. The

IgG response in intestine must result from a paracellular diffusion of serum-derived and locally produced IgG through epithelia. The IgA responses, generally lower than IgG responses, were induced by subcutaneous route. Antigenicity of recombinant PsaA protein could be confirmed by the results of these subcutaneous immunizations.

(5-2) Enzyme-linked immunosorbent assay(ELISA)

The samples were analyzed in duplicate for detection of anti-PsaA antibodies by ELISA. Polystyrene microtiter plate(Corning©) was coated with 3.2 μg/ml PsaA in bicarbonate coating buffer(40 mM Na_2CO_3, 60 mM $NaHCO_3$, pH9.6). After an overnight adsorption at 4 °C, the contents of the plates were washed twice with PBS containing 0.05 % Tween 20© (PBST). The wells were filled with a 5 % skim milk solution and incubated for 2 hrs at 37 °C for blocking. The contents were discarded again, and the wells were rinsed twice with PBST. Serum specimens were diluted 50 times, and gut wash and bronchoalveolar lavage samples diluted 2 fold were used as primary antibodies. The secondary antibodies used were horseradish peroxidase(HRP)-conjugated goat anti-mouse immunoglobulin (OrganonTeknika Corp., USA) diluted 2,000 times. All immunoglobulins used were diluted in PBS containing 1 % skim milk and incubated at 37 °C for 30

min. After each step, the wells were washed 6 times with PBST. All reagents were used in a standard volume of 100 μl. Stable peroxidase substrate buffer(PIERCE Chemical Co., USA) containing OPD(1 mg/ml) was used to develop color. After the reaction was stopped by adding 100 μl of 2.0 N sulfuric acid, the optical density(O.D.) at 492 nm was measured using a microplate reader(Molecular Device Corp, USA). The sample was assayed in duplicate and a pair of blank well was included on each plate.

The quantity of the antibodies is expressed as geometric mean of concentraion of antibodies in the samples. For the determination of the concentration of antibodies, standard curves were made using sandwich ELISA. Micro-titer plates were coated with 50 μl of bicarbonate buffer containing 4 μg of goat anti-mouse immunoglobulin isotypes(IgM, IgG, IgA; Organon Teknika Corp. Durham, NC, USA) overnight at 4 °C. After blocking with 5 % skim milk, purified mouse immunoglobulin(Organon Teknika Corp.) with known concentration of isotypes served as a standard.

Optical density was proportional to IgM antibody concentration from 20 to 120 ng/ml, to mouse IgG antibody at concentraions from 10 to 110 ng/ml and to mouse IgA antibody at concentraions from 10 to 100 ng/ml(see Fig.12).

(5-3) Systemic and local antibody responses to PsaA with formulation for oral administration

The antigens were resuspened in PBS with 3 % Na_2Co_3 immediately before peroral(P.O.) immunization with 40 ug/mouse of E (PsaA) or N (PsaA). The mice were orally administered with the antigen solution(500 μl/dose) via blunt-tipped feeding needle inserted into the stomach.

At 2 weeks after triple immunizations of mice with N(PsaA) or E(PsaA), PsaA specific antibody responses at serum, gut wash and lung lavage obtained using method of step (5-1) were assayed according to method of step (5-2). The effect of the carrier on the immunogenicity of orally-administered PsaA was investigated by comparing the immune responses of the mice immunized with E(PsaA) and N(PsaA)(see Fig.13).

E(PsaA) induced prominent IgG, and IgA responses at serum, bronchoalveolar sites, and intestine among these groups (p value < 0.05), suggesting that the encapsulated antigen enhance both the systemic and the mucosal antibody responses probably by the protection of antigens against degradation.

(5-4) Immune responses to PsaA enhanced by cholera toxin

To determine whether cholera toxin(CT) can

modulate systemic and mucosal immune responses against PsaA, CT was co-administered with PsaA. Co-administration of CT with E (PsaA) significantly enhanced the systemic and the mucosal IgA responses of mice (p value<0.05). Oral administration of mucosal adjuvant CT with microencapsulated antigen not only induced specific serum antibody responses to CT, but also was proven to be an effective regimen for the generation of antigen-specific antibody responses in mucosal sites. Through oral immunization of microencapsulated PsaA with CT, both systemic and mucosal immune responses were significantly enhanced.

Cholera toxin B subunit(CTB) enhanced serum IgG production when co-administered with antigens. But it had no enhancement effect on mucosal immune responses in this example, even though CTB plays a role as a mucosal adjuvant in a conjugated fashion(Bergquist C, 1995).

INDUSTRIAL APPLICABILITY

The microspheres of this invention are considered as a proper carrier for an efficient oral delivery of antigens because antigens can be incorporated into alginate microcapsules that protect antigens from denaturation at low pH and hydrolytic degradation by

protease in gastrointestinal tract.

As the microsphere of this invention is less than 5 μm in diameter, therefore, it can be effectively deliver the incoporated antigens to the Peyer's patch and to lymphatics in systemic and local immune systems via concomitant transport pathways. Additionally, the microsphere of this invention not only shows higher levels of antigenicity than the naked protein antigen but is to enhance the level of serum and mucosal antibodies when co-administered orally with cholera toxin or cholera toxin B subunit, which are known to be powerful mucosal adjuvants.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is claimed is

1. A vaccine composition for oral administration consisting essentially of a protein antigen, in an amount effective to induce an immune response to said antigen, encapsulated in alginate microspheres.

2. The vaccine composition of claim 1, wherein said protein antigen is one or more selected from the group comprising bacterial surface protein antigens, endotoxin or exotoxin antigens, autoantigens, and allergens.

3. The vaccine composition of claim 1, wherein said protein antigen is one or more selected from the group comprising a pnemococcal antigen, a *Hemophilus parainfluenza* antigen, a diphtheria antigen, a pertussis antigen, a tetanus antigen, a enterotoxigenic *E. coli* antigen, a dysentery antigen, a cholera antigen, a gonococcus antigen, a influenza virus antigen, B type hepatitis virus antigen, a measles antigen, a smallpox virus antigen, and a rubella antigen.

4. The vaccine composition of claim 3, wherein said pneumococcal protein antigen is one or more selected from group comprising pneumolysin, neuraminidase,

autolysin, pneumococcal surface adhesion A(PsaA), and pneumococcal surface protein A(PspA).

5. The vaccine composition of claim 1, wherein said vaccine includes additionally immnune adjuvants such as cholera toxin, cholera toxin B subunit, *Escherichia coli* Heat labile enterotoxin, muramyldipeptide or phorbolester.

6. The vaccine composition of claim 5, wherein said immune adjuvant is added to inside or outside of the alginate microsphere.

7. The vaccine composition of claim 1, wherein the alginate microsphere is between 0.1 to 5 μm in diameter.

8. The vaccine composition of claim 1, wherein said vaccine causes mucosal immunity response.

9. A process for preparing the vaccine composition of claim 1, comprising the steps of:

a) Mixing aliquot of a protein antigen or a protein antigen including immune adjuvant with a alginate aqueous solution;

b) Homogenizing by adding a mixture of the alginate and a protein antigen of step a) to n-octanol

containing an emulsifier;

c) Spraying n-octanol solution containing $CaCl_2$ into the emulsion while stirring the whole emulsion slowly;

d) Adding additional $CaCl_2$ solution to saturate the emulsion, and curing microspheres;

e) Dehydrating microspheres by addition of dehydrating solvent;

f) Collecting the microspheres on membrane filters and washing with alcohol, and then drying *in vacuo*.

10. The process of claim 9, wherein said protein antigen is one or more selected from the group comprising bacterial surface proteins, endotoxin or exotoxin antigens, autoantigens and allergens.

11. The vaccine composition of claim 9, wherein said protein antigen is one or more selected from the group comprising a pnemococcal antigen, a *Hemophilus parainfluenza* antigen, a diphtheria antigen, a pertussis antigen, a tetanus antigen, a enterotoxigenic *E. coli* antigen, a dysentery antigen, a cholera antigen, a gonococcus antigen, a influenza virus antigen, B type hepatitis virus antigen, a measles antigen, a smallpox virus antigen, and a rubella antigen.

12. The vaccine composition of claim 11, wherein said pneumococcal protein antigen is one more selected from group comprising pneumolysin, neuraminidase, autolysin, pneumococcal surface adhesion A(PsaA), and pneumococcal surface protein A(PspA).

13. The process of claim 9, wherein said vaccine includes additionally immnune adjuvants such as cholera toxin, cholera toxin B subunit, *Escherichia coli* Heat labile enterotoxin, muramyldipeptide or phorbolester.

14. The process of claim 13, wherein said immuine adjuvant is added to inside or outside of the microspheres.

15. The process of claim 9, wherein the alginate microspheres solution of step a) is used in 1-5 weight%.

16. The process of claim 9, wherein the emulsifier of step b) is selected from the group comprising HCO-10, HCO-60 and Span-80 which is used in 1-10 weight%.

17. The process of claim 9, wherein the n-octanol containing $CaCl_2$ in step c) is used in 0.5-7 weight%.

18. The process of claim 9, wherein the $CaCl_2$ solution of step d) is used in 5-10 weight%.

19. The process of claim 9, wherein the dehydrating solvent of step e) is methanol, ethanol, isopropanol or acetone.

20. The process of claim 9, wherein the filter of step f) is a polyvinylidene difluoride(PVDF) membrane filter with a pore size between 0.10-0.44 μm.

21. The process of claim 9, wherein the alginate microsphere is approximately 0.1 to 5 μm in diameter.

FIG. 1

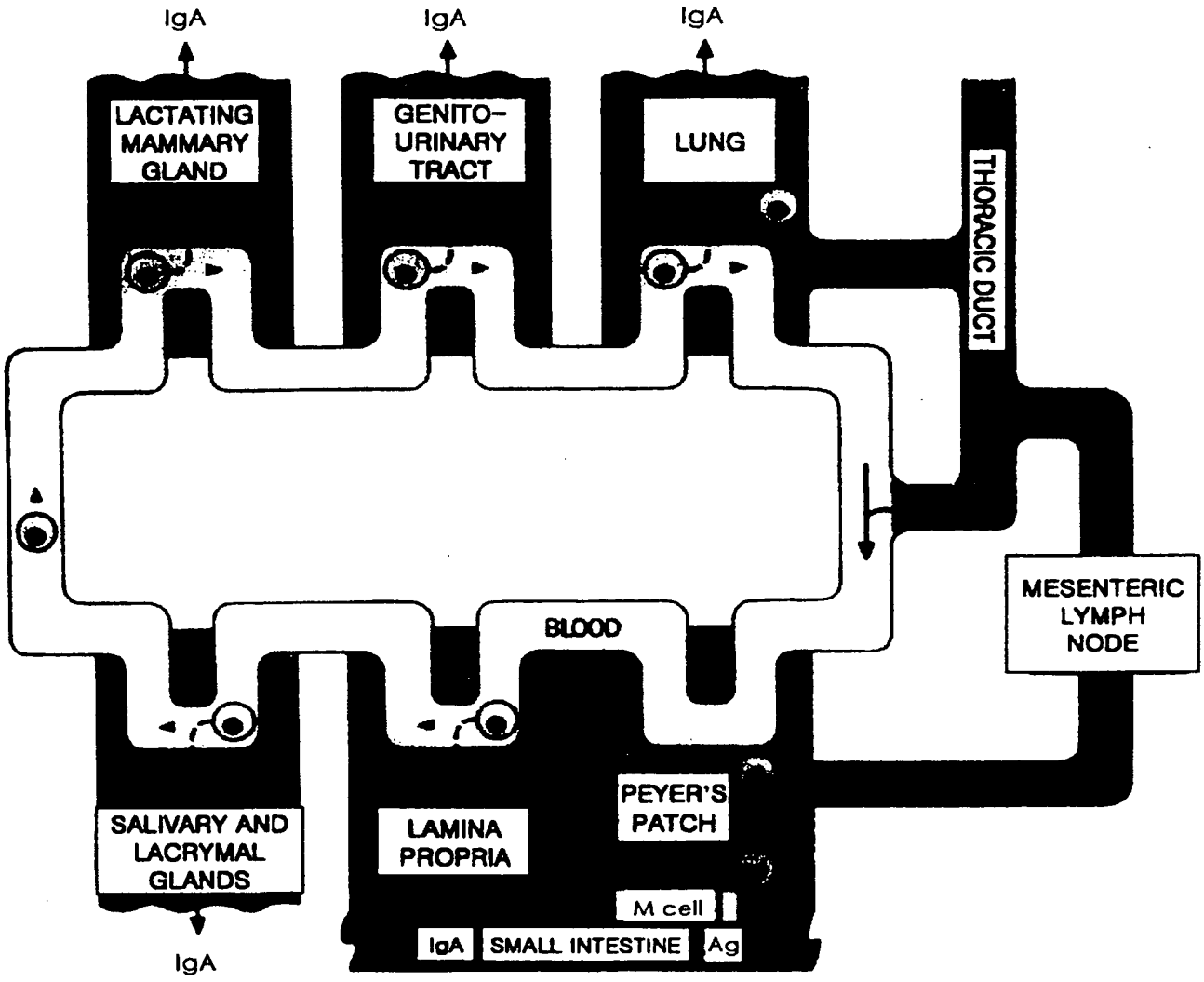
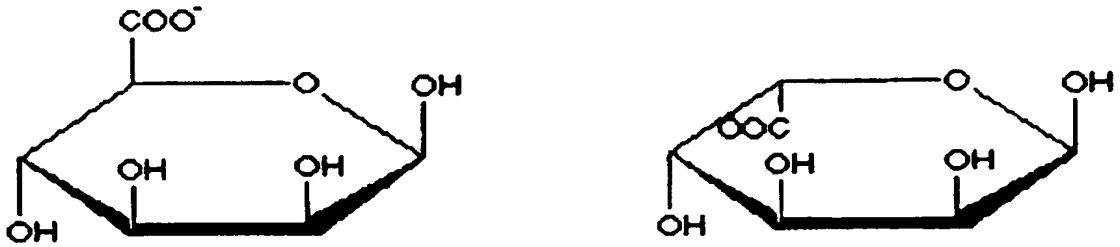


FIG. 2a



(M) (G)

FIG. 2b

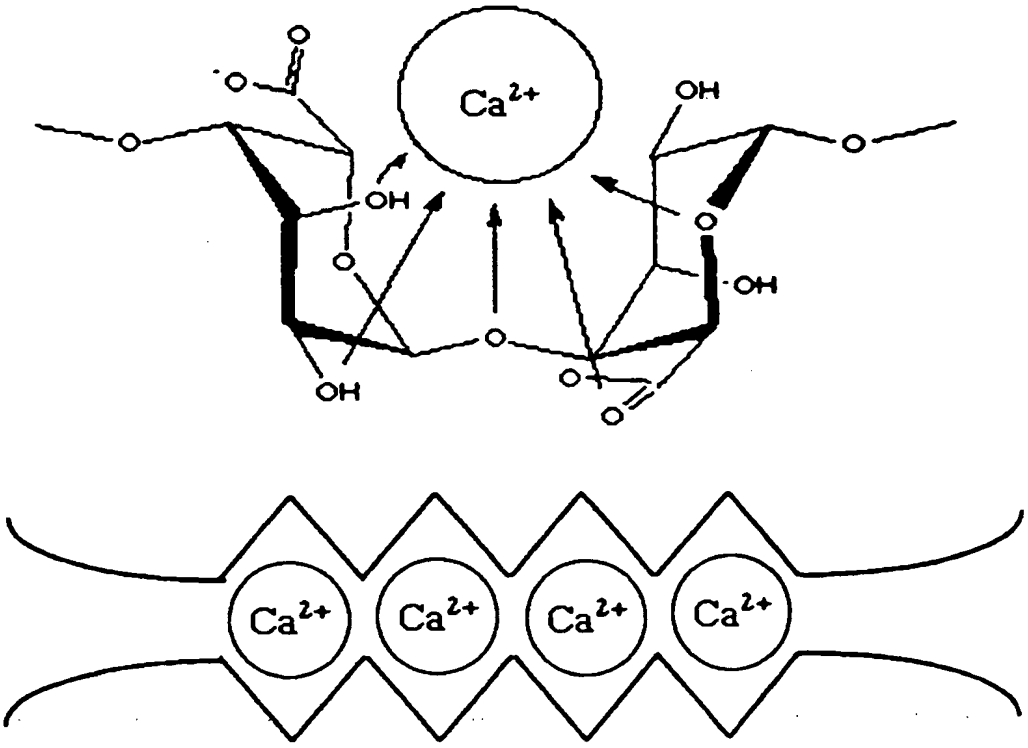


FIG. 3

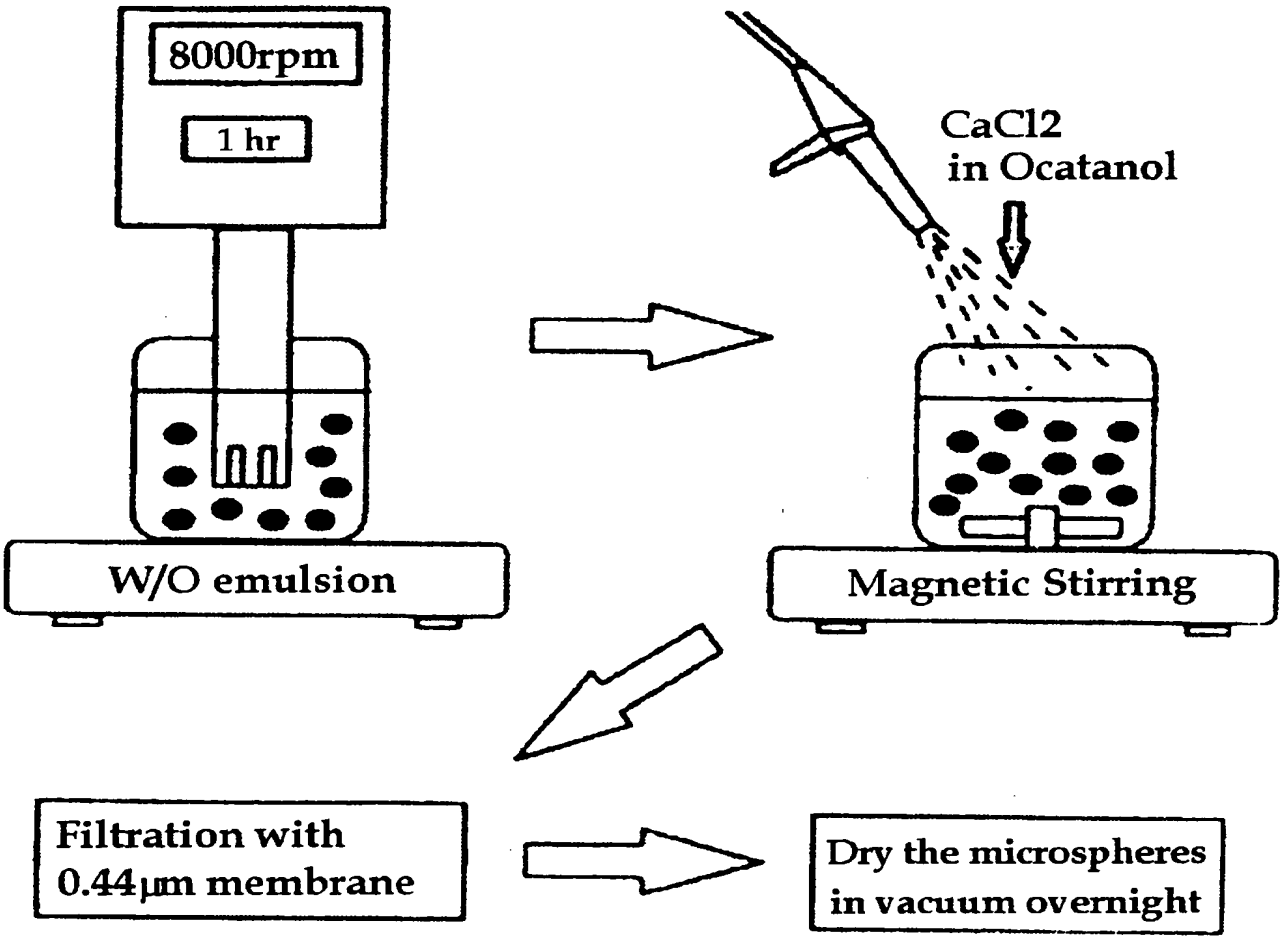


FIG. 4

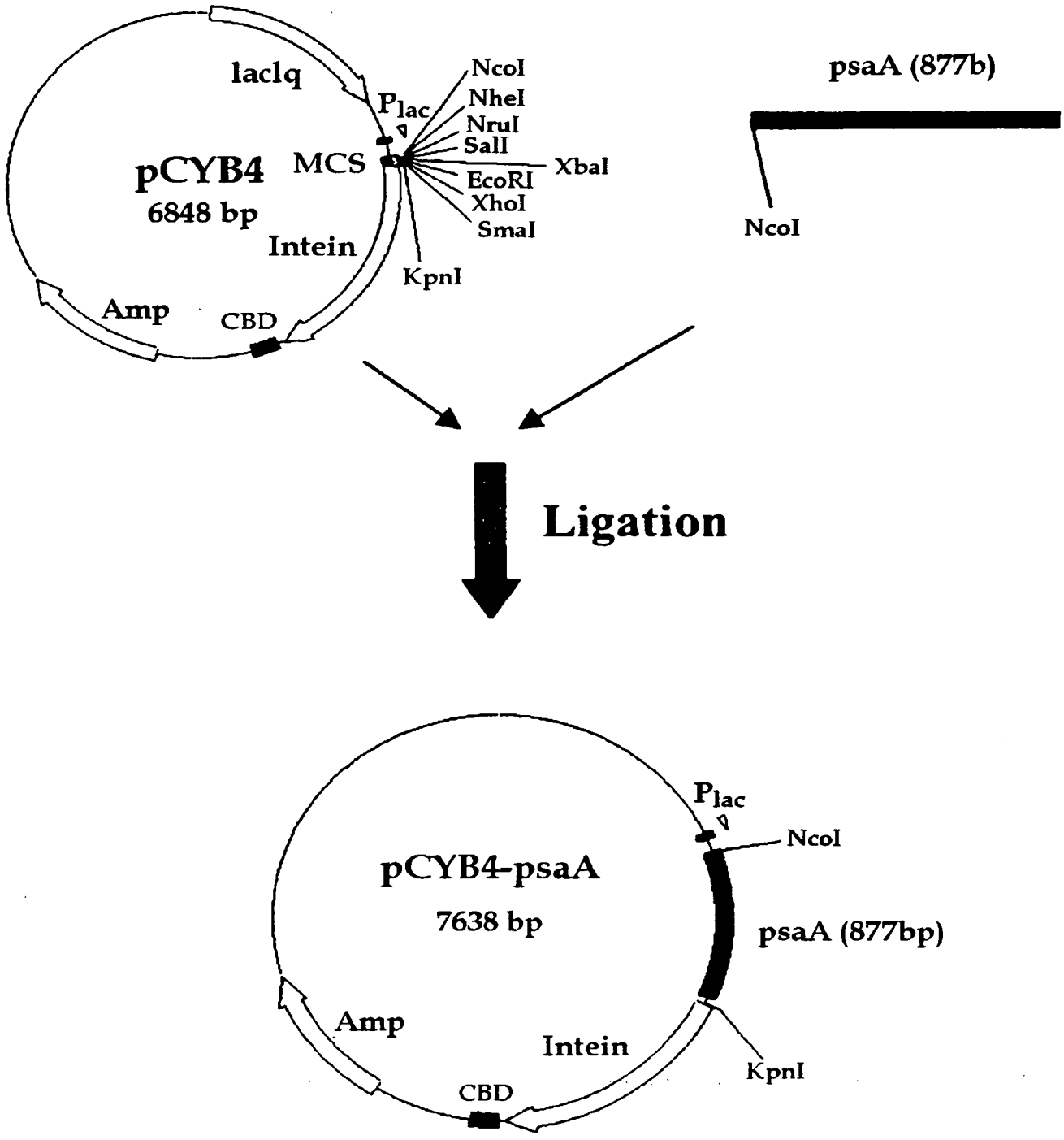


FIG. 5

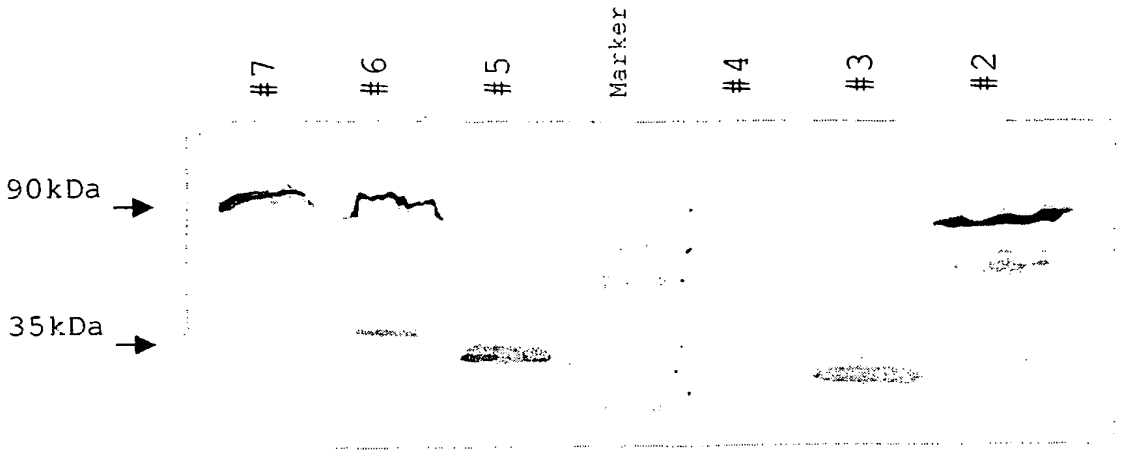


FIG. 6

161-GCAAAGCCCTAATAAATTGG

RBS

181-AGGATCTAATGaaaaaatta

Start codon

201-ggtacattactcgttctctt

signal peptide

GenBank :221-tctttctgcaatcattcttg

Clonig site *ACAAGGACCATAGACCATGG*

pCYB4 ——— NcoI

241-tagcatgtGCTAGCGGAAAA

TAGCATGTGCTAGCGGAAAA

541-TCAGCGACGGCGTTGATGTT

TCAGCGACGGCGTTG----

691-TCTATGAAAAAAATCTCAAA

----TGAAAAAAATCTCAAA

1101-GAAGGATTGGCAAAATAAGC

Stop codon

GAAGGATTGGCAG*GGTGCTT*---

pCYB4 —

1311-ATTTTTCTCTTACTACAACA-1330

FIG. 7

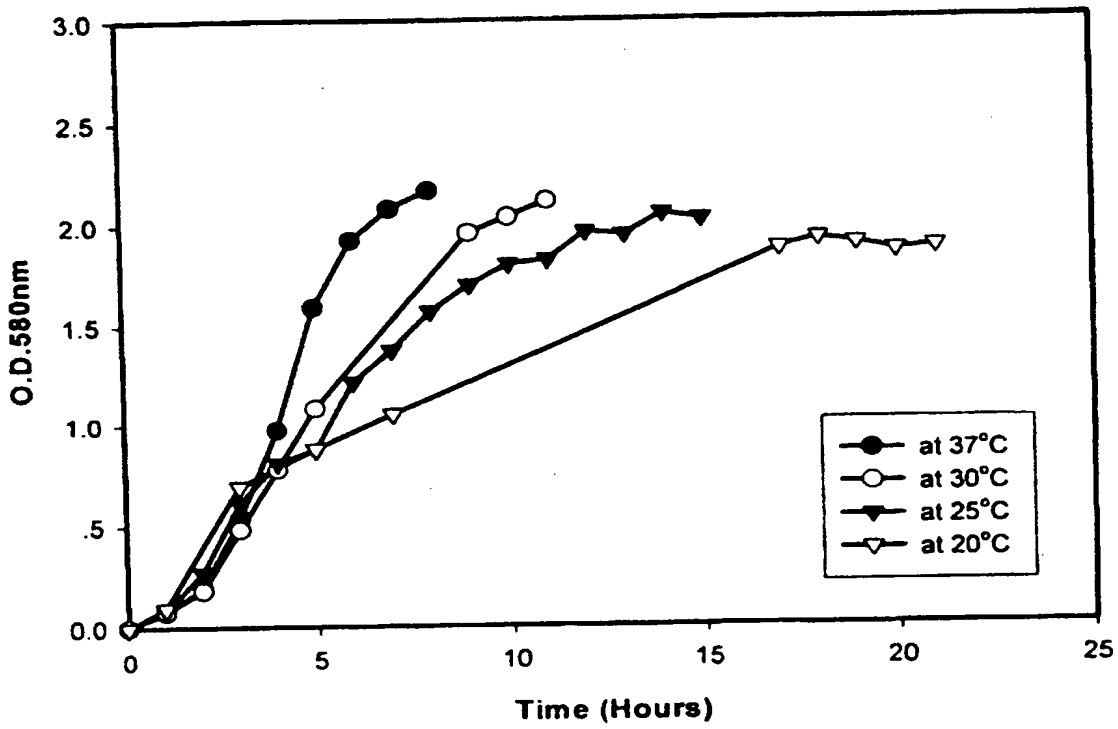


FIG. 8a

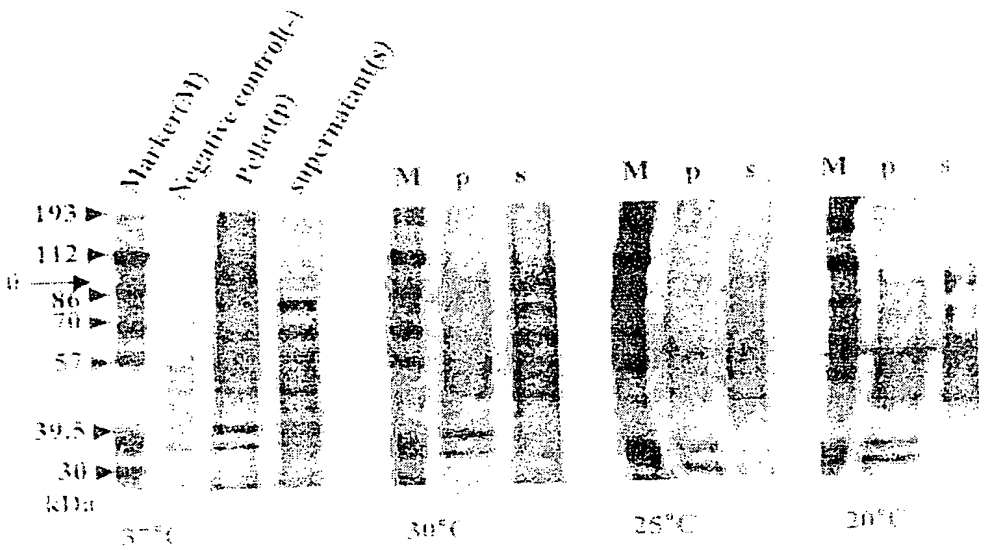


FIG. 8b

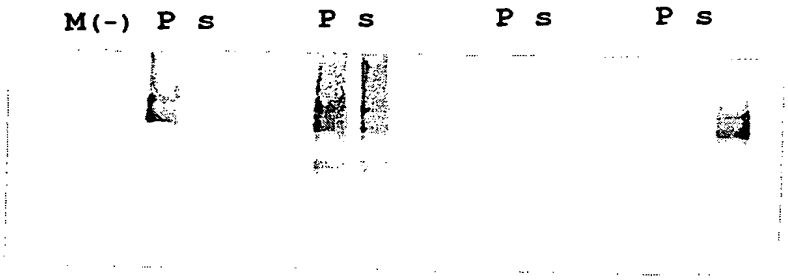


FIG. 9a

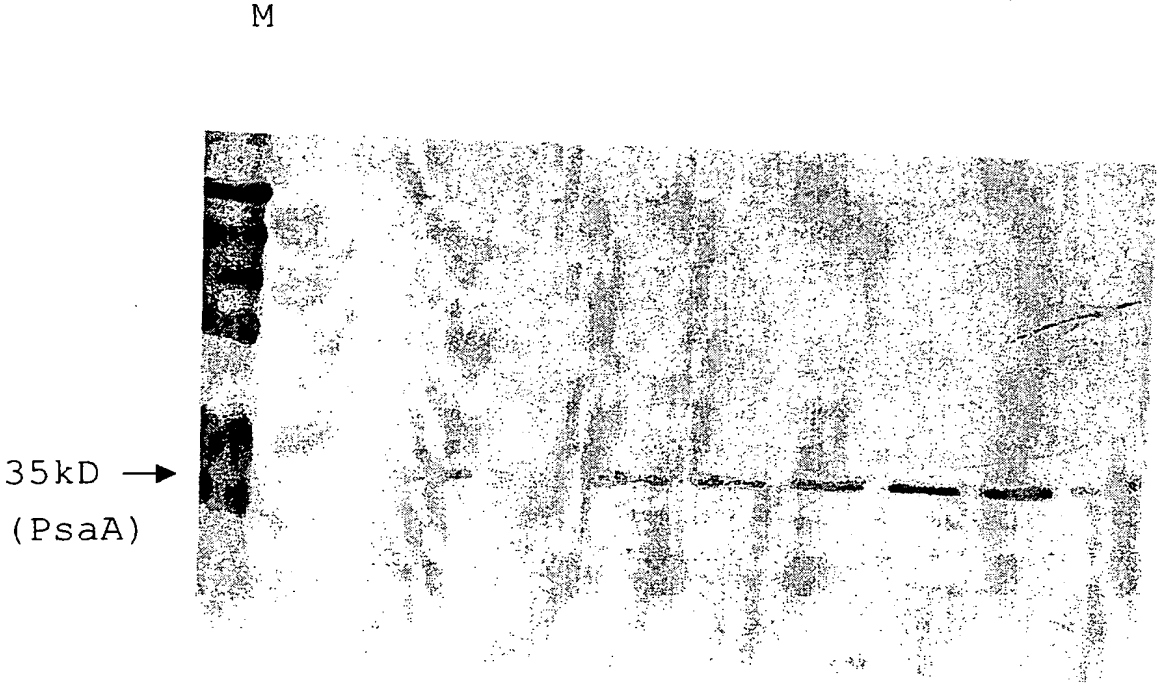


FIG. 9b

FIG. 10

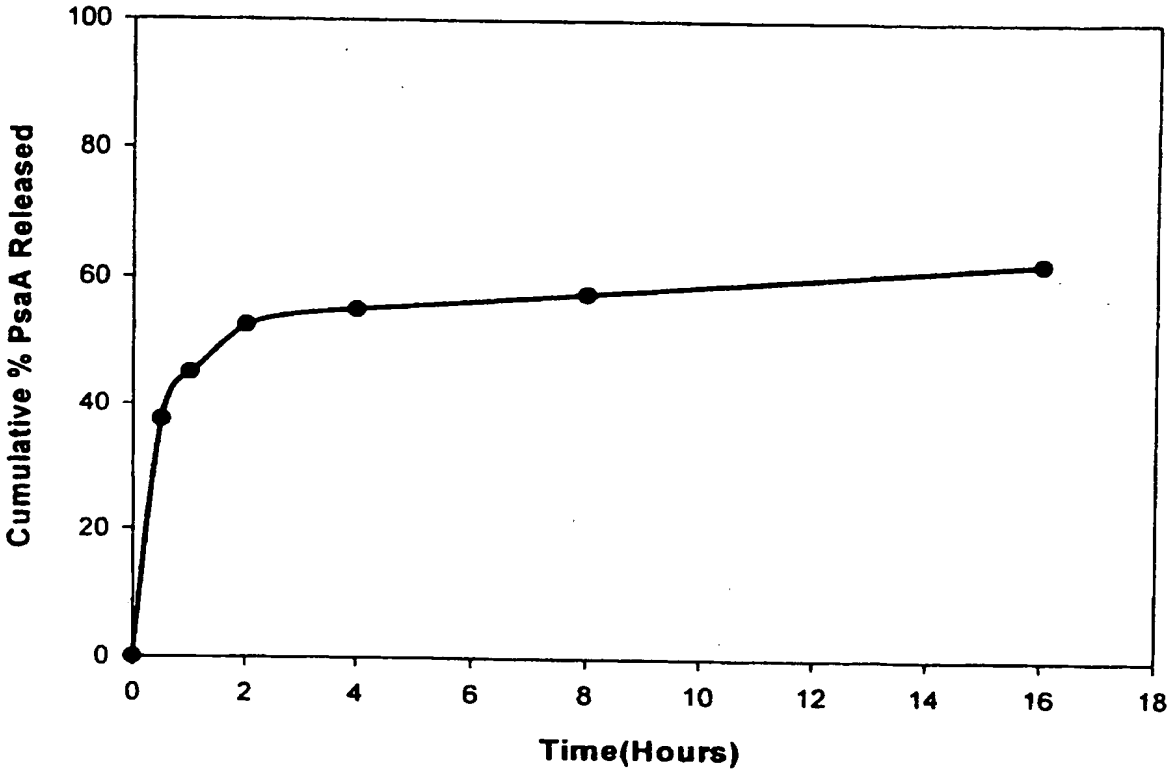


FIG. 11

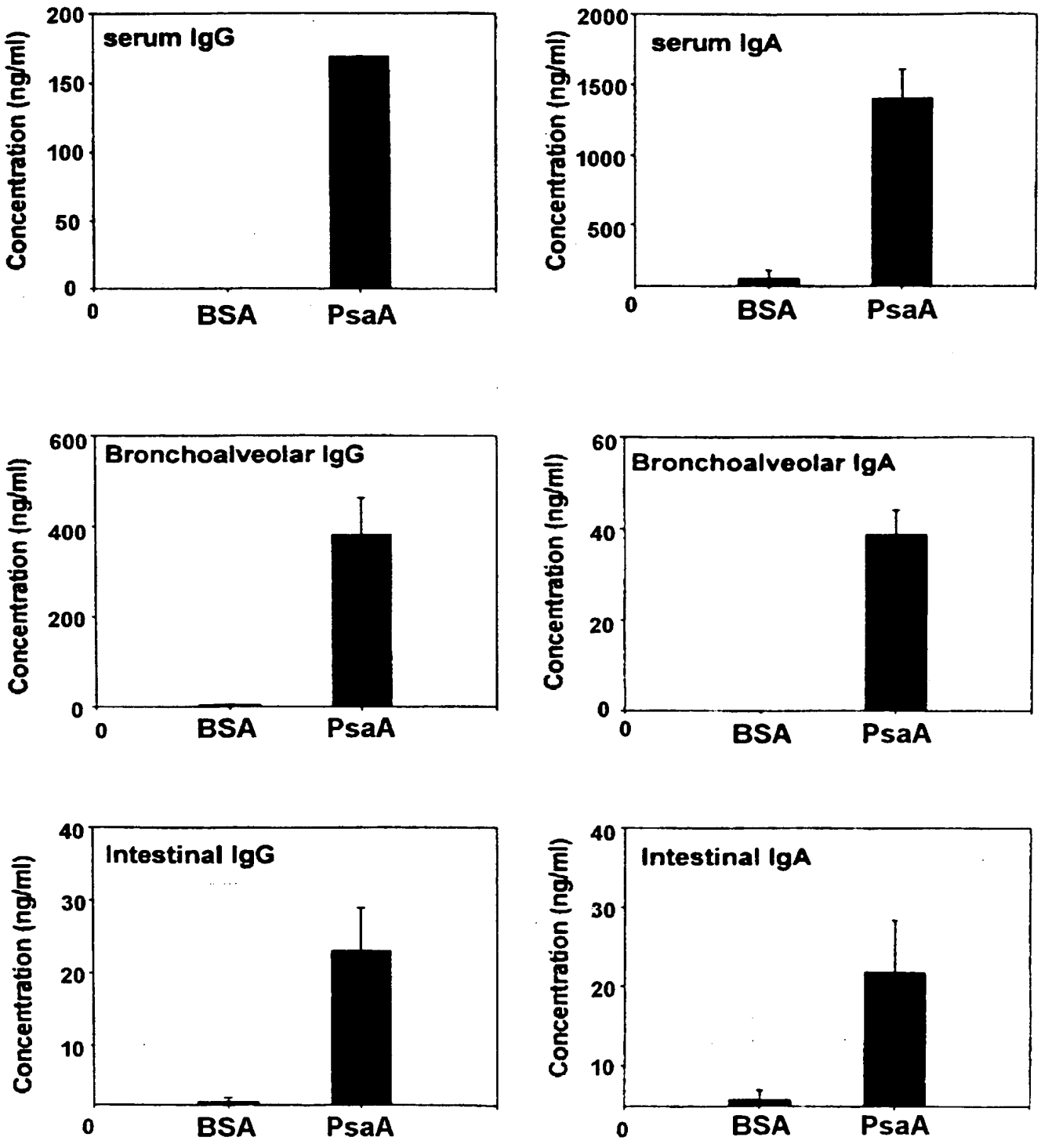
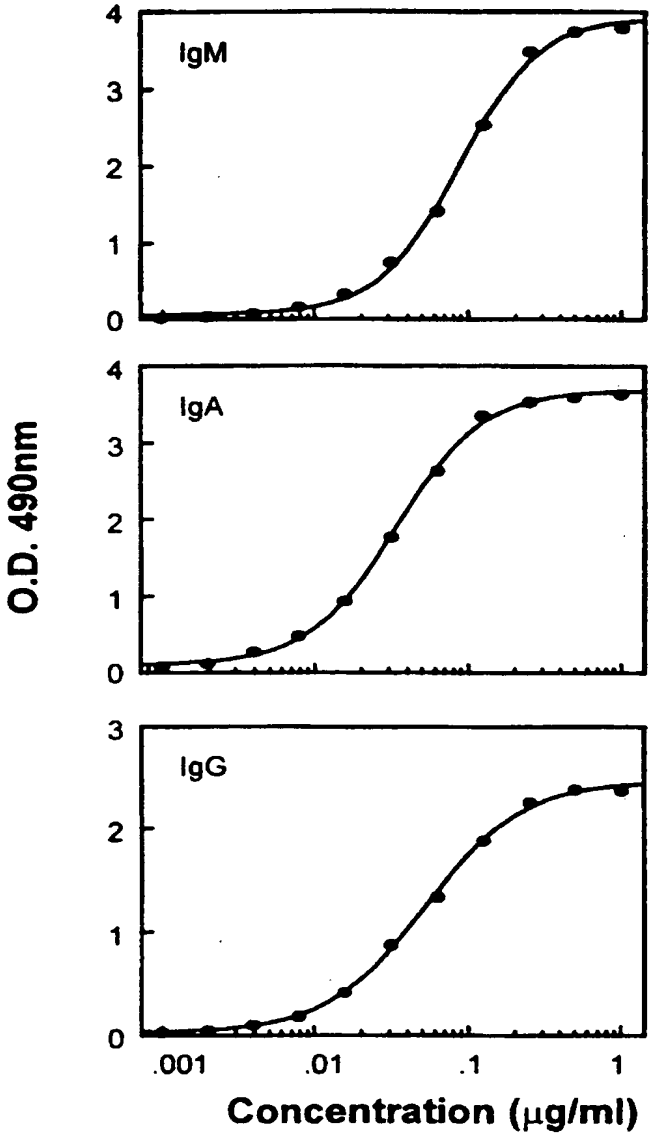


FIG. 12



O.D.=((A-D)/(1+(CONC./C)^B))+D
A: 0.064
B: 1.673
C: 0.086
D: 3.923
R^2: 0.998
CONC.=C*((A-D)/(O.D.-D)-1)^(1/B)

O.D.=((A-D)/(1+(CONC./C)^B))+D
A: 0.018
B: 1.354
C: 0.052
D: 2.464
R^2: 0.999
CONC.=C*((A-D)/(O.D.-D)-

O.D.=((A-D)/(1+(CONC./C)^B))+D
A: 0.096
B: 1.541
C: 0.034
D: 3.681
R^2: 0.999
CONC.=C*((A-D)/(O.D.-D)-1)^(1/B)

FIG. 13

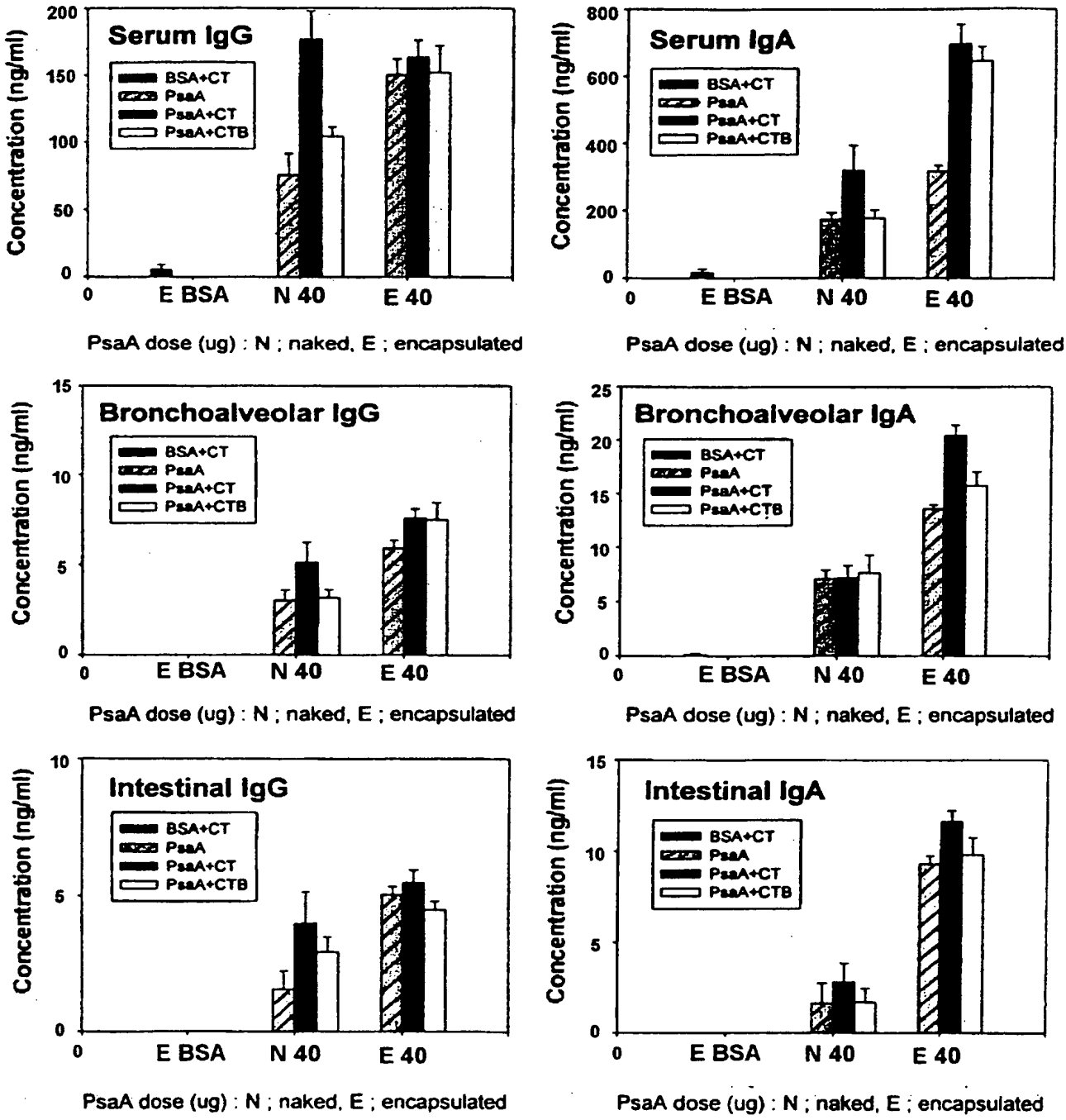
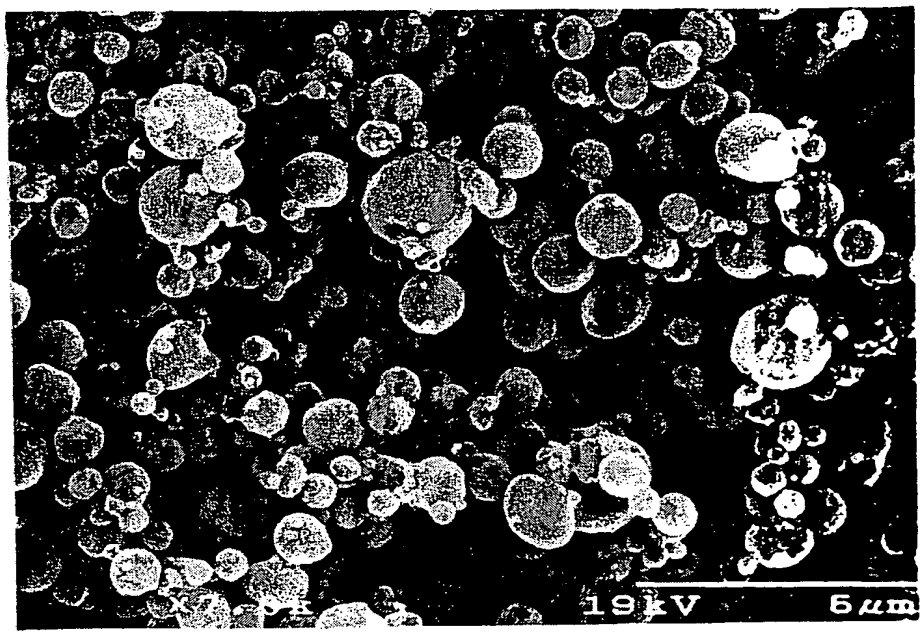


FIG. 14



SEQUENCE LISTING

<110> Korea Institute of Science and Technology

<120> Vaccine compositions using antigens encapsulated within alginate microspheres for oral...

<130> 9FPO-06-12

<150> KR 99-24336
<151> 1999-06-25

<160> 4

<170> KOPATIN 1.5

<210> 1
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI-tagged sense primer for PsaA genomic DNA

<400> 1
tcaccatggt agcatgtgct agcgg 25

<210> 2
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense primer for PsaA genomic DNA

<400> 2
tgccaatcct tcagcaatct tgtca 25

<210> 3
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> sense primer for Ptac promotor

<400> 3
gagcggataa caatttcaca cagg 24

<210> 4
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense primer for intein

<400> 4
gaggttggta ataaggtcat gggt 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00466

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7]: A 61 K 39/00, 38/16, 47/36, 9/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: A 61 K 39/00, 38/16, 47/36, 9/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5900238 A (GOMBOTZ et al.) 04 May 1999 (04.05.99), columns 3-11; claims.	1-3, 5, 6, 8
X	WO 98/18346 A2 (PRIVATES INSTITUT BIOSERV GMBH), abstract; page 4, lines 14-32; page 6, lines 2-9; example 6; claims 1, 10, 11, 15.	1-3
A	EP 873752 A2 (PFIZER INC.) 28 October 1998 (28.10.98), page 3, lines 37-51; page 12, line 51 - page 15, line 36.	1-8

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2000 (22.02.00)	04 April 2000 (04.04.00)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Authorized officer Mosser Telephone No. 1/53424/437

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 99/00466

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
US	A	5900238	04-05-1999			none	
EO	A	9818346a2				none	
EP	A2	873752	28-10-1998	AU	A1	63511/98	12-11-1998
				AU	B2	713639	09-12-1999
				CA	AA	2229430	23-10-1998
				CN	A	1196931	28-10-1998
				JP	A2	10316586	02-12-1998
				NZ	A	330251	25-11-1998